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Proteolysis of SNAP-25 by Types E and A Botulinal Neurotoxins*

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Clostridial neurotoxins, tetanus toxin (TeTx) and the seven related but serologically distinct botulinal neurotoxins (BoNT/A to BoNT/G), are potent inhibitors of synaptic vesicle exocytosis in nerve endings. Recently it was reported that the light chains of clostridial neurotoxins act as zinc-dependent metalloproteases which specifically cleave synaptic target proteins such as synaptobrevin/VAMPs, HPC-1/syntaxin (BoNT/C1), and SNAP-25 (BoNT/A). We show here that BoNT/E, like BoNT/A, cleaves SNAP-25, as generated by *in vitro* translation or by expression in *Escherichia coli*. BoNT/E cleaves the Arg^{180}-Ile^{181} bond. This site is different from that of BoNT/A, which cleaves SNAP-25 between the amino acid residues Gln^{197} and Arg^{198}. These findings further support the view that clostridial neurotoxins have evolved from an ancestral protease recognizing the exocytotic fusion machinery of synaptic vesicles whereby individual toxins target different members of the membrane fusion complex.

Clostridial neurotoxins are generated as single-chain polypeptides (M_r 150,000) that are proteolytically activated into the dichain toxins in which the L[1] chains (M_r 50,000) remain linked to the H chains (M_r 100,000) via a disulfide bond (Niemann, 1991). In contrast to BoNT/A, which is produced by a proteolytic strain and thus released as an active di-chain toxin, BoNT/E is released from a nonproteolytic strain as a single-chain derivative (DasGupta and Rasmussen, 1983). After uptake into the host organism BoNT/E is activated by host proteases. Activation can also be achieved *in vitro* by means of trypsin incubation (Sathyamoorthy and DasGupta, 1985). The H chains mediate neuroselective binding, internalization, intraneuronal sorting, and finally translocation of the corresponding L chains from an acidic compartment into the cytosol. Here, the L chains act as metalloproteases that specifically cleave synaptic proteins that are directly involved in the vesicular release of neurotransmitters. Thus TeTx, BoNT/B, BoNT/D, and BoNT/F hydrolyze synaptobrevin isoforms (Schiavo *et al.*, 1992, 1993; Link *et al.*, 1992).[2] BoNT/C1 specifically degrades HPC-1/syntaxin (Blasi *et al.*, 1993a), and BoNT/A proteolyzes the synaptic protein SNAP-25 (Blasi *et al.*, 1993b). Söllner *et al.* (1993) have reported that synaptobrevin, syntaxin, and SNAP-25 form a complex that binds the cytosolic proteins NSF and α-, β-, γ-SNAPs. The latter proteins were shown to be required for various intracellular fusion events in which NSF acts as an ATPase (Rothman and Orci, 1992). In this study, we demonstrate that BoNT/E cleaves SNAP-25 between Arg^{180} and Ile^{181}. This site differs from the BoNT/A cleavage site shown here to be the Gln^{197}-Arg^{198} bond.

EXPERIMENTAL PROCEDURES

Neurotoxins—BoNT/A was isolated from the Hall strain according to Sugii and Sakaguchi (1975). For production of BoNT/E, the Beluga strain was used following the procedure of DasGupta and Rasmussen (1983). For proteolytic activation, single-chain BoNT/E (500 nM final concentration) in 500 μl of 50 mM sodium acetate buffer, pH 6.0, containing 0.2 M NaCl, was incubated for 30 min at 37 °C under shaking in the presence of 280 milliunits of agarose-immobilized trypsin (Pierce Chemical Co.). After incubation, soybean trypsin inhibitor (5 μM, final concentration; Boehringer Mannheim) was added, the agarose beads were removed by two consecutive centrifugation steps (10,000 × *g*, 10 min), and activated BoNT/E was recovered in the supernatant. BoNT/E and BoNT/A were reduced by incubation for 30 min at 37 °C in 10 mM (final concentration) dithiothreitol.

Bacterial Expression of SNAP-25 and Digestion with BoNT/E and BoNT/A—A clone encoding SNAP-25 was isolated from a rat brain cDNA library by PCR using the two primers 5′-CGCGGATCCATGGC(C/G)GA(A/G)GACGC(A/G)GA(C/T)ATG-3′ and 5′-CGCAGATCTAACCACT(T/G)CC(C/G)AGCATCTT-3′. The amplified fragment was digested with *Bam*HI and *Bgl*II, cloned into the *Bam*HI site of pGEX-2T (Pharmacia LKB Biotechnology Inc.), and sequenced by the chain termination method (Sanger *et al.*, 1977). The deduced amino acid sequence was identical to the mouse SNAP-25 sequence published previously (Oyler *et al.*, 1990). For *in vitro* transcription/translation, the SNAP-25 gene was recovered as a *Bam*HI-*Eco*RI fragment and cloned into pSP72 (Promega, Heidelberg, Germany).

For expression of an authentic SNAP-25 polypeptide, we first introduced a singular *Hpa*I blunt-end site following the last codon of the SNAP-25 open reading frame. As an upstream primer in the PCR, we used 5′-CTCTGAATTCGCAATTAAGGAGATAATAGGTATGGCCGAGGACGCAGACATGCGT-3′. This primer provided the *Eco*RI site for cloning and a Shine-Dalgarno sequence for ribosomal binding. 5′-CTCTCGTCGACGTTAACACCACTTCCCAGCATCTTTGTTGCAC - 3′, providing the *Hpa*I site, was used as the 3′-primer. A set of two complementary oligonucleotides encoding the sequence PPTPGHHHHHH and a translational termination signal was inserted into the *Hpa*I site. Recombinant clones were verified by DNA sequencing. To express SNAP-25, we transferred the *Eco*RI-*Sal*I fragment into pQE3 (Diagen, Düsseldorf, Germany). Induction and isolation of the recombinant SNAP-25 protein via nickel-nitrilotriacetic acid agarose (Ni-NTA-agarose, Diagen) were performed according to the manufacturer's instructions. For digestion with reduced activated BoNT/E or BoNT/A, 30 μg of purified SNAP-25 (or its 9.5-kDa C-terminal fragment) were incubated

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[1] The abbreviations used are: L and H chains, light and heavy chains, respectively; TeTx, tetanus toxin; BoNT/A–G, botulinal neurotoxins A–G; VAMP, vesicle-associated membrane protein (acronyms for synaptobrevin 1 and 2); SNAP-25, synaptosome-associated protein of 25 kDa; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF-attachment protein; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction.

[2] S. Yamasaki, A. Baumeister, T. Binz, J. Blasi, E. Link, F. Cornille, B. Roques, E. M. Fykse, T. C. Südhof, R. Jahn, and H. Niemann, submitted for publication.

for 60 min at 37 °C in 50 µl of 20 mM HEPES/KOH, pH 7.0, containing 100 mM NaCl and 200 nM (final concentration) activated reduced BoNT/E or BoNT/A. Aliquots were analyzed on 15% SDS-polyacrylamide gels according to Laemmli (1970).

Other Methods—SNAP-25 peptides and cleavage products were isolated by reverse phase chromatography using a Nucleosil 5-µm C8 column (250 × 4 mm) from Macherey and Nagel (Düren, Germany). Amino acid sequences were determined on a model 473A protein sequencer from Applied Biosystems (Foster City, CA).

In Vitro Transcription / Translations—Radiolabeled SNAP-25 was obtained as a substrate for BoNT/E and BoNT/A applying the combined TNT™-coupled reticulocyte lysate system from Promega according to manufacturer's instructions. For *in vitro* synthesis of the BoNT/E L chain, we amplified the corresponding gene fragment using the two primers 5′-CACAGGATCCATGCCAAAAATTAATAGTTTTAA-3′ and 5′-CTCTCAAGCTTAGGCCTTATGCCTTTTACAGAAAC-3′ and chromosomal DNA (Poulet *et al.*, 1992) as template. The fragment was cut with *Bam*HI and *Hind*III and cloned under control of the SP6 promoter into pSP73 (Promega). pBN4, encoding the entire L chain of BoNT/A under control of the SP6 promoter (Binz *et al.*, 1990), was used for production of BoNT/A L chain-specific mRNA.

RESULTS AND DISCUSSION

We have recently identified SNAP-25 as the substrate of BoNT/A (Blasi *et al.*, 1993b). Comparative studies on the action of BoNT/A and BoNT/E on the vertebrate neuromuscular junction demonstrated that both types of neurotoxin act in a similar manner with respect to blocking spontaneous miniature endplate potentials (Molgo *et al.*, 1989, 1990). Therefore, we examined whether BoNT/E, like BoNT/A, proteolyzes SNAP-25. For this purpose, we generated radiolabeled SNAP-25 by combined *in vitro* transcription/translation. As shown in Fig. 1*A* (*lane 3*), a major translation product was generated that exhibited an apparent molecular mass of 28 kDa, similar to that of native SNAP-25. This translation product was then incubated with BoNT/E L chain that was generated by *in vitro* transcription/translation from the cloned gene (Poulet *et al.*, 1992) in the absence of radiolabeled amino acids. As shown in Fig. 1*A* (*lane 4*) incubation with recombinant BoNT/E L chain resulted in the generation of a product of smaller molecular weight (M_r shift approximately 3,000) demonstrating that SNAP-25 is proteolyzed. In a parallel incubation, SNAP-25 was also incubated with recombinant L chain of BoNT/A. In agreement with our previous observations (Blasi *et al.*, 1993b), this also resulted in cleavage of SNAP-25 (*lane 4*). However, the major fragment generated by BoNT/A L chain was larger than that generated by BoNT/E L chain, indicating that the respective cleavage sites are different. Together our data demonstrate that both BoNT/E and BoNT/A hydrolyze SNAP-25. Because the BoNT/E L chain was generated by *in vitro* transcription/translation of cloned DNA, a contamination by foreign proteases is excluded.

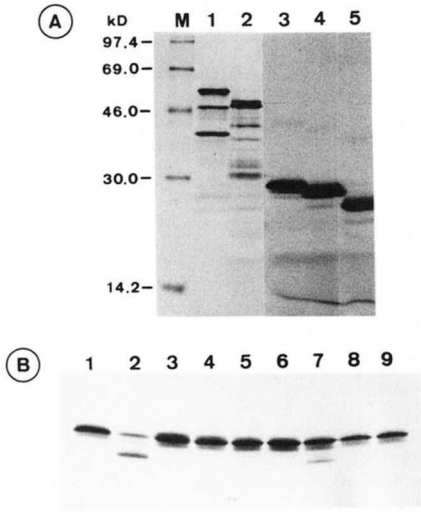

FIG. 1. **Cleavage of SNAP-25 by BoNT/E and BoNT/A.** *A*, cleavage by recombinant BoNT/E and BoNT/A L chains. SNAP-25 was translated in the presence of [^{35}S]methionine and incubated for 60 min at 37 °C with aliquots of BoNT/E (*lane 5*) or BoNT/A (*lane 4*) that were generated by *in vitro* transcription/translation but in the absence of labeled amino acids. To control for the expression of the BoNT/E and BoNT/A L chains, translation of the L chains was also performed in the presence of [^{35}S]methionine, resulting in the appearance of major translation products of the expected size (*lane 1*, BoNT/A L chain; *lane 2*, BoNT/E L chain). Note that in addition to SNAP-25, an unidentified translation product of lower mobility is generated. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on 15% gels and processed by autoradiography. *B*, cleavage of SNAP-25 by trypsin-activated BoNT/E derived from *C. botulinum*. *In vitro* translated SNAP-25 was incubated at 37 °C for 60 min either alone (*lane 1*) or with the following additions: *lane 2*, BoNT/E (40 nM final concentration); *lane 3*, BoNT/E + 20 mM dipicolinic acid (*DPD*); *lane 4*, BoNT/E + 20 mM *o*-phenanthroline (*PTL*); *lane 5*, BoNT/E + 20 mM EDTA; *lane 6*, heat-inactivated BoNT/E (10 min at 100 °C); *lane 7*, BoNT/E + 5 µM soybean trypsin inhibitor; *lane 8*, 22.5 milliunits of trypsin; *lane 9*, trypsin (22.5 milliunits) treated with 5 µM soybean trypsin inhibitor. Samples were separated on a 12% polyacrylamide gel, and bands were visualized by autoradiography.

In the next experiments, we studied whether SNAP-25 was also cleaved when BoNT/E was used that was isolated from *Clostridium botulinum* and activated by trypsin-containing beads prior to the incubation. Again, radiolabeled SNAP-25 generated by *in vitro* translation was used as a substrate. As shown in Fig. 1*B* (*lane 2*), activated BoNT/E generated a cleavage product of SNAP-25 that was of a size similar to that observed after incubation with the recombinant L chain. No cleavage of SNAP-25 was observed even when it was directly incubated with trypsin beads (regardless of whether soybean trypsin inhibitor was present or not; *lanes 8* and *9*), indicating that BoNT/E-induced cleavage is not due to residual trypsin in the incubation mixture. In contrast, BoNT/E cleavage of SNAP-25 was still observed even when excess amounts of soybean trypsin inhibitor were present (*lane 7*). However, BoNT/E-induced proteolysis of SNAP-25 was abolished in the presence of various metal ion chelators or when the toxin was heat-inactivated (*lanes 3–6*). Together these data show that trypsin-activated BoNT/E, as purified from *C. botulinum* was free of contaminating proteases and could thus be used for determination of the cleavage site.

To obtain larger amounts of substrate, we engineered a SNAP-25 gene that encoded a C-terminal His_6 tag preceded by a unique IgA-protease cleavage site (PPTP; Pohlner *et al.* (1992)). This mutant gene was expressed in *E. coli* M15 under control of a tightly regulated promoter. The His tag allowed purification of recombinant SNAP-25 by chromatography on Ni-NTA-agarose. As shown in *lane 1* of Fig. 2, the largest molecular species in the eluate fraction corresponded in size to that of the full-length recombinant polypeptide. Furthermore, microsequencing revealed that it contained the authentic N-terminal sequence originally reported by Oyler *et al.*, (1989). This material was further purified by reverse phase HPLC (*lane 2*) and used as a substrate for trypsin-activated and reduced BoNT/E (*lane 4*) and reduced BoNT/A (*lane 3*). The size of the individual larger sized cleavage products correlated with that previously observed in the *in vitro* approach. Furthermore, these fragments failed to bind to the Ni-NTA-agarose, indicating that both toxins cleaved peptide bonds close to the C-terminal end (data not shown). The *arrowheads* point to positions of the small C-terminal fragments, which, however, were barely detectable on the Coomassie Blue-stained gel.

In addition to full-length SNAP-25, some lower molecular weight species co-purified on the Ni-NTA-agarose column, indicating that they represented C-terminal fragments carrying the His_6 tag. These peptides were not obtained when the recombinant *E. coli* cells were lysed with 6 M guanidinium hydrochloride (not shown). The smallest of them (M_r 9,500) was also

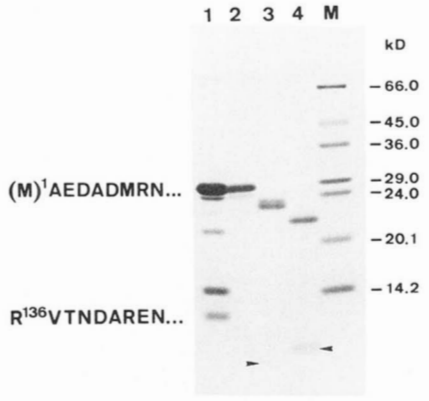

FIG. 2. **Cleavage of *E. coli*-expressed SNAP-25 by BoNT/E and BoNT/A.** Recombinant *E. coli* cells expressing the SNAP-25 gene under control of an isopropyl-1-thio-β-D-galactopyranoside-inducible promoter were lysed under physiological conditions, and SNAP-25 carrying a C-terminal His_6 tag was bound via Ni-NTA-agarose according to the protocols of the manufacturer. *Lane 1* shows that full-length SNAP-25 and a set of C-terminal fragments were eluted from Ni-NTA agarose. The subfragments were subsequently purified by reverse phase HPLC. Microsequencing of the largest (full-length) and smallest polypeptides yielded the sequences indicated on the *left*. Full-length SNAP-25 was digested with purified reduced BoNT/A (*lane 3*) and with reduced BoNT/E, previously activated with trypsin (*lane 4*). Samples were analyzed on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue. *Arrowheads* indicate the positions of the C-terminal peptides released by the individual L chains. Molecular mass markers are shown on the *right*.

isolated by HPLC and microsequenced. The N-terminal sequence indicated that it originated from proteolytic degradation affecting the Arg^{135}-Arg^{136} bond. This molecular species was cleaved as efficiently as full-length SNAP-25.

Taking into account that the presence of the C-terminal His_6 tag does not interfere with proteolysis, our data suggest that neither BoNT/E nor BoNT/A require the presence of the authentic N and C termini. We have shown recently that TeTx, BoNT/D, and BoNT/F cleave rat synaptobrevin 2 at different sites.[2] Each of the toxins required about 10 residues at the C-terminal side of the scissile bond. In each instance, however, optimum cleavability required the presence of more distal N-terminal sequences.[2] It will be interesting to see whether BoNT/E and BoNT/A, cleaving SNAP-25 at different peptide bonds, have related substrate requirements. In this context, it is of interest that the N-terminal fragment generated from SNAP-25 by cleavage with BoNT/A still serves as a substrate for BoNT/E (data not shown).

For isolation of the C-terminal cleavage products, we applied reverse phase HPLC. *Panel A* of Fig. 3 shows the elution profile obtained for full-size untreated SNAP-25. Upon cleavage with BoNT/E (*panel B*), the parental peak was shifted from 27.33 to 27.03 min and a new peak eluting at retention time of 13.32 min appeared. Material underlying this peak was collected. Microsequencing over 10 cycles produced the sequence IMEKADSNKT. Thus, BoNT/E cleaves SNAP-25 at the Arg^{180}-Ile^{181} bond.

In addition, we collected the C-terminal fragment generated by treatment with BoNT/A (*panel C*). This fragment eluted after 11.52 min from the HPLC column. Microsequencing yielded the sequence RATKMLGSGV, whereby the valine residue stems from the introduction of the *Hpa*I site originally used to introduce the His_6 tag. Thus, BoNT/A cleaves SNAP-25 at the Gln^{197}-Arg^{198} bond, releasing only a nonapeptide as a C-terminal cleavage product.

In contrast to the other targets of clostridial neurotoxins (synaptobrevins and syntaxins), SNAP-25 does not contain a transmembrane anchor domain but associates with membranes through palmitoylation of 1 or more of the 4 cysteine residues between positions 82 and 92 (Hess *et al.*, 1992). Therefore, cleavage with BoNT/E and BoNT/A will not affect membrane association of SNAP-25. Since both BoNT/E and BoNT/A efficiently block exocytotic release of neurotransmitters, our data suggest a direct role of the C-terminal domain of SNAP-25 in this process. Whereas it is unlikely that cleavage of SNAP-25 by BoNT/A results in a dramatic change of the tertiary structure (the residual fragment is still cleaved by BoNT/E), it is likely that the C-terminal domain interacts with other proteins. Recently, Söllner *et al.* (1993) have reported that SNAP-25, synaptobrevin, and HPC-1/syntaxin constitute the essential components of a docking-fusion machinery that binds other soluble cytosolic proteins, NSFs and α, β, γ-SNAPs, in an ATP-dependent manner. Both NSFs and SNAPs are associated with this complex in a well defined stoichiometric ratio (Wilson *et al.*, 1992) and have been shown earlier to be required for a series of intracellular fusion events (Rothman and Orci, 1992). It is intriguing to speculate that the clostridial neurotoxins have evolved from one ancestral gene encoding a protease that selectively cleaved synaptobrevin and thus blocked neurotransmitter release. During evolution, the individual neurotoxins have diverged, some of them (TeTx/BoNT/B, BoNT/D, and BoNT/F) cleaving still the same substrate, however at different peptide bonds. Yet other clostridial neurotoxins have developed specificity for other members of the same exocytotic protein

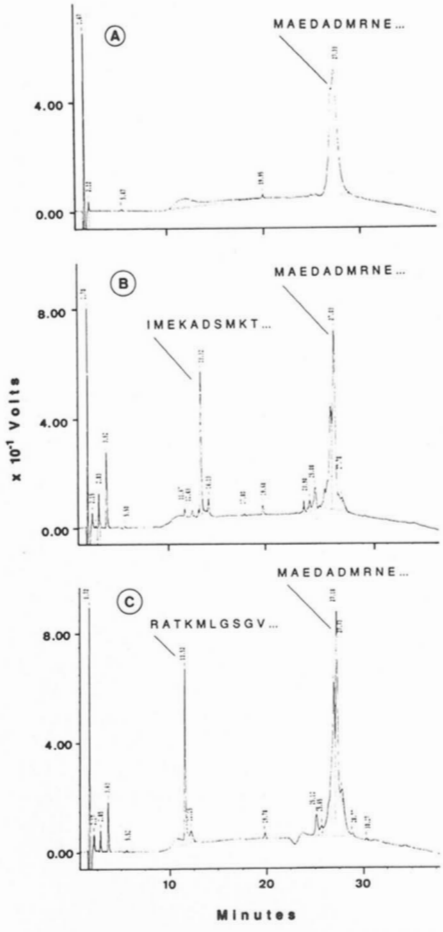

FIG. 3. **HPLC profiles of SNAP-25 before and after digestion with BoNT/E and BoNT/A.** HPLC-purified full length SNAP-25 was incubated for 60 min at 37 °C alone (*A*), in the presence of 200 nM trypsin-activated, reduced BoNT/E (*B*) or 200 nM reduced BoNT/A (*C*). Samples were analyzed by reverse phase HPLC. Peak fractions were collected, and underlying material was subjected to microsequencing. Amino acid sequences specify the N-terminal sequences of the fragments.

complex. Thus BoNT/C1 hydrolyzes HPC-1/syntaxin, whereas BoNT/E and BoNT/A degrade SNAP-25, again at different sites. The detection of cellubrevin, a TeTx-sensitive synaptobrevin homologue present in all eukaryotic cells examined (McMahon *et al.*, 1993), suggests that a similar protein complex regulates constitutive exocytosis in all eukaryotic cells. It will be interesting to see whether HPC-1/syntaxin or SNAP-25 isoforms can be detected in these cells as substrates for BoNT/C1, BoNT/A, and BoNT/E.

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